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## ORIGINAL INVESTIGATION

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## Exon skipping in the sterol 27-hydroxylase gene leads to cerebrotendinous xanthomatosis

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**Abstract** We report a new mutation in the sterol 27-hydroxylase (CYP 27) gene in a Dutch family with cerebrotendinous xanthomatosis: a G→A transition in the splice donor site in intron 4. This mutation leads to skipping of exon 4, resulting in a loss of 66 amino acids in the CYP 27 enzyme molecule.

### Introduction

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive lipid storage disease characterised by a disturbed bile acid metabolism caused by a deficiency of the mitochondrial enzyme, sterol 27-hydroxylase (CYP 27). Deficiency of this enzyme leads, among other things, to an excessive production of cholestanol that accumulates in many tissues (Björkhem and Boberg 1995). The clinical spectrum of CTX is characterised by premature bilateral cataract, followed by neurological and neuropsychiatric abnormalities and formation of tendon xanthomas

(Björkhem and Boberg 1995). In children, the combination of bilateral cataract and intractable diarrhoea is indicative of CTX (Cruysberg et al. 1991).

The cDNA which encodes the rabbit mitochondrial CYP 27, a member of the mitochondrial cytochrome P450 enzyme family, was elucidated by Andersson et al. 1989. The cDNA of the human CYP 27 has been cloned and its gene localised on chromosome 2 (q33-qter) (Cali and Russell 1991). The structure of the human CYP 27 gene was elucidated by Leitersdorf et al. (1993).

In the Netherlands, 39 CTX patients from 18 families have been identified up to the present. This report describes a new splice site mutation in the CYP 27 gene in the Dutch CTX population, a mutation which was found in three patients from one family.

### Materials and methods

#### Subjects

The clinical and biochemical characteristics of the patients (from non-consanguineous parents) are described in Table 1. The difference in phenotypic expression between the three siblings is remarkable. An intractable diarrhoea, a symptom rarely reported in adult CTX patients, was present in patient A. Clinical signs and symptoms were most severe in patient B: she had severe Parkinsonism, a syndrome that has been previously reported in CTX patients (Rogelet et al. 1992; Shibata et al. 1990). The frequently described cerebellar signs were absent in patient C.

#### Biochemical analysis

The cholestanol and cholesterol levels in serum and the urinary excretion of bile alcohols were measured according to Wolthers et al. (1983, 1991) using capillary gas chromatography.

#### Mutation detection and restriction analysis

The CYP 27 gene was amplified in four fragments (exons 1, 2, exons 3–5 and exons 6–9), by PCR from genomic DNA of leucocytes. Exons 3–9 with their intron boundaries were subsequently amplified separately, with the two PCR fragments 3–5 and 6–9 as templates (Luyten et al. 1995). The oligonucleotides used as

The nucleotide sequence data reported here have been deposited with the Genome Database and are available under the accession number G00-700-058

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primers for PCR amplification and for sequence analysis are those mentioned in a publication by Leitersdorf et al. (1993). The amplimers thus obtained were subjected to single-strand conformation polymorphism (SSCP) analysis using the Pharmacia Phast

system. Cycle sequencing of the coding and the non-coding strands of all amplimers was carried out by the Taq Dye Deoxy Terminator method using an ABI 377 DNA sequencer.

RNA (5 µg) from cultured fibroblasts was reverse transcribed to cDNA in 1 h at 42°C with 200 U Superscript II reverse transcriptase (Life Technologies, Breda, The Netherlands), using oligo(dT) and random hexamer primers. PCR amplification of the cDNA exon 3–exon 6 region was performed according to established procedures (Ploos van Amstel et al. 1996). The oligonucleotides used as primers were: forward primer, 5'-GGAAGGA-CACCACTGGTACCAGC-3' (cDNA position 468–480) in exon 3, reverse primer, 5'-CGCAGAGTCTCCTTAAGCACAGC-3' (cDNA position 1205–1183) in exon 6. The amplimer obtained was used for agarose gel electrophoresis and for DNA sequence analysis, as described above.

**Table 1** Clinical characteristics and serum cholesterol and cholesterol levels of the patients at the time of diagnosis (+ mild, ++ moderate, +++ severe, – absent)

	Patient		
	A	B	C
Sex	Female	Female	Male
Age (years)	51	48	44
Age at diagnosis (years)	44	41	37
Major systematic signs			
Cataract	++	++	++
Achilles tendon xanthomas	++	++	++
Diarrhoea	++	–	–
Neurological signs			
Low intelligence/dementia	+	+++	+
Epilepsy	+	+	+
Parkinsonism	–	+++	–
Dysarthria	–	+++	–
Pyramidal signs	+	++	+
Cerebellar signs	+	++	–
Dorsal column involvement	+	++	+
Peripheral neuropathy	+	+	+
Other systemic signs			
Coronary artery disease	–	–	–
Pulmonary disease	–	–	–

**Results**

SSCP analysis of the amplimer of exon 4 displayed bands in the three patients which were not present in the controls. In the SSCP of the parents, this pattern was present, together with that of a control amplimer (Fig. 1 A). Direct sequencing of exon 4 demonstrated a G→A transition at position 865 + 1 (numbering according to Cali and Russell 1991). This mutation was confirmed by means of restriction enzyme analysis with *HphI*, which cleaves the normal amplimer of exon 4 into three fragments of 245, 40 and 12 bp, and the amplimer with the mutation into two fragments of 285 and 12 bp (Fig. 1 B). In 58 controls, this mutation was not found.

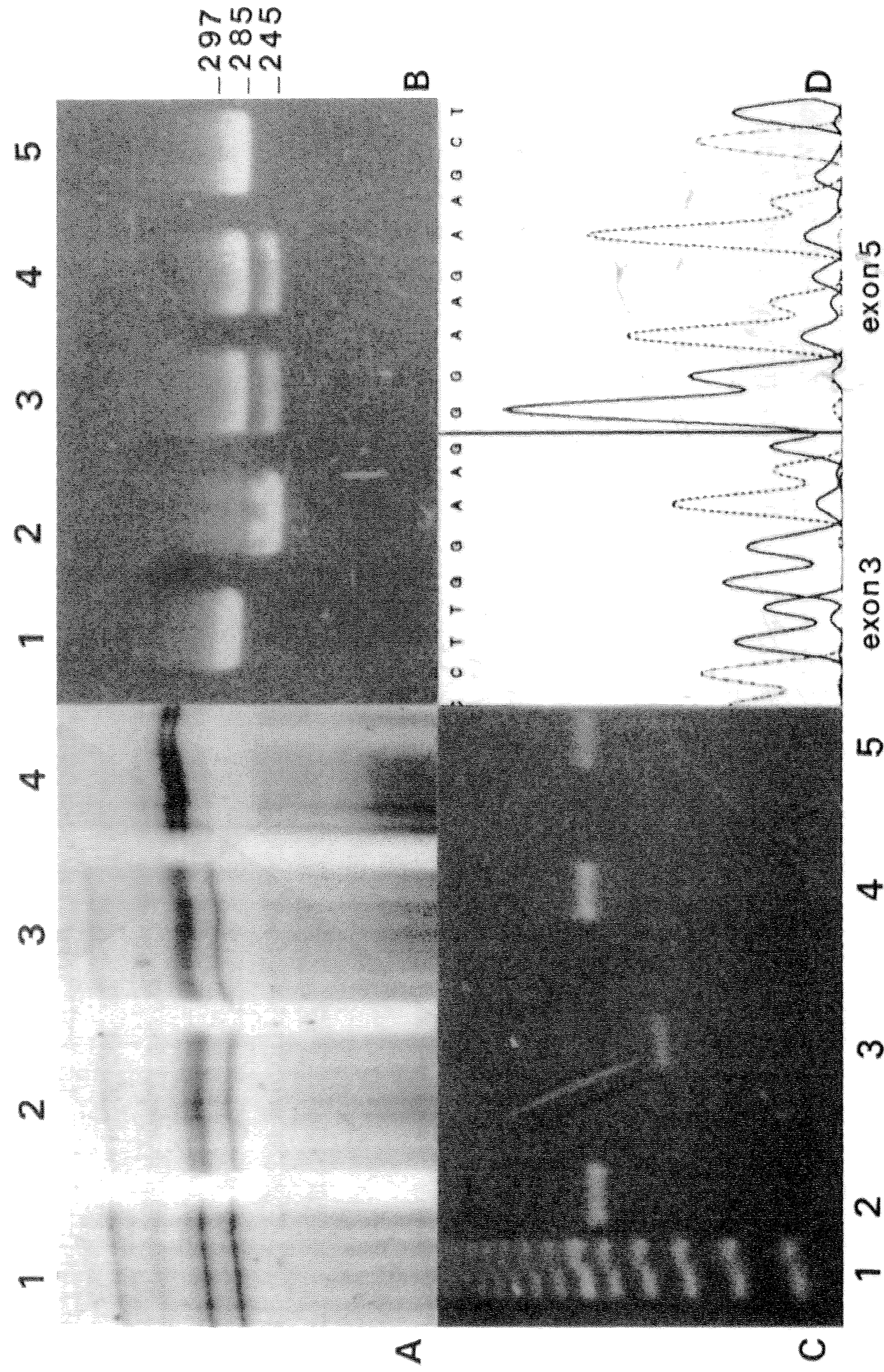
The functional significance of the splice site mutation was further analysed by agarose gel electrophoresis of the



cDNA exon 3–exon 6 amplicmer. The cDNA amplicmer of patient B was smaller than that of the wild-type cDNA (Fig. 1 C). A skipping of exon 4 resulting in a conjunction of exon 3 and exon 5, was found by sequencing this amplicmer (Fig. 1 D).

Osteomalacia	–	–	–	–	–
Biochemical findings					
Serum cholestanol (3.3–12.5 $\mu\text{mol/l}$ )	33	80	83		
Serum cholesterol (2.6–5.2 $\text{mmol/l}$ )	2.4	4.4	3.9		

**Fig. 1** A Single-strand conformation polymorphism analysis (12.5% polyacrylamide gel, 4°C, 400 V, 5 mA, 1 W, 300 Vh) of the amplicmer of exon 4. *Lane 1* control, *lane 2* mother, *lane 3* father, *lane 4* patient. **B** Polyacrylamide (6%) gel electrophoresis of the amplicmer of exon 4. *Lane 1* control before digestion with *HphI*; *lane 2* control, *lane 3* mother, *lane 4*, father, *lane 5* patient amplicmer after digestion with *HphI*. **C** Agarose (2%) gel electrophoresis of exon 3–exon 6 cDNA of patient B. *lanes 2, 4 and 5* control amplicmer, *lane 3* amplicmer of patient B, *lane 1* 100-bp marker. **D** Sequence of the cDNA exon 3–exon 5 conjunction area of the exon 3–exon 6 amplicmer





## Discussion

In this study, three clinically well-characterised CTX patients from one family were investigated for mutations in the CYP 27 gene.

Several mutations have previously been reported in CTX patients. Three mutations in splice sites affecting normal splicing have been found (Garuti et al. 1996; Leitersdorf et al. 1993). In the present communication, we present a novel splice site mutation: a G→A transition at cDNA position 865 + 1 (intron 4), resulting in skipping of exon 4. This leads to a loss of 198 bp in the mRNA and of 66 amino acids in the enzyme molecule. The patients were homozygous for this mutation.

The loss of 66 amino acids in the enzyme molecule must lead to a considerable deficiency in the enzyme activity. The late onset of the disease in these patients is an indication that this part of the enzyme molecule is not pivotal for its activity. This is in accordance with the finding that the conserved part of the gene is located in the exon 6–exon 8 region (Leitersdorf et al. 1993). The presence of this splice site mutation in the homozygous form results in an intrafamilial phenotypic variability (Table 1), which points to a contribution of other factors to the phenotypic expression. As the phenotype of CTX is highly variable, even within a family with the same mutation, it is important to search for the relevant mutation in family members with atypical signs and symptoms.

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